MOLECULAR AND CELLULAR BIOLOGY, Nov. 1988, p. 4821–4828
0270-7306/88/114821-08$02.00/0

Vol. 8, No. 11

A *Mup* Promoter-Thymidine Kinase Reporter Gene Shows Relaxed Tissue-Specific Expression and Confers Male Sterility upon Transgenic Mice

RAYA AL-SHAWI,[1] JOANNE BURKE,[1] CHERYL T. JONES,[1] J. PAUL SIMONS,[2] AND JOHN O. BISHOP[1]*

Department of Genetics, University of Edinburgh, Edinburgh EH9 3JN,[1] and AFRC IAPGR-ERS, Edinburgh EH9 3JQ,[2] Scotland

Received 22 June 1988/Accepted 9 August 1988

A hybrid gene was made by fusing the 2.2-kilobase 5′ promoter region of a mouse group 1 major urinary protein (*Mup*) gene to the coding region of the herpes simplex virus type 1 thymidine kinase gene (HSV *tk*) and introduced into the genomes of mice by microinjection. Transgenic G_0 males were sterile, or when fertile did not transmit the foreign gene, and the transgenic male descendants of G_0 females were also sterile. Seven "lines" were established by breeding from G_0 females and their transgenic female descendants. Six lines expressed HSV thymidine kinase activity in the liver, and activity correlated perfectly with the presence of HSV *tk* RNA. In three of four lines examined, expression was lower in female than in male liver, and in these lines the same sex difference was observed in the rate of run-on transcription of the foreign genes in liver nuclei. When females of one of the sexually dimorphic lines were treated with testosterone, the levels of HSV *tk* RNA and thymidine kinase activity were increased, although not to male levels. In these aspects of liver expression, and also in a lack of expression in seven other tissues, the hybrid gene exhibits many of the characteristics of an endogenous group 1 *Mup* gene. However, the gene was also expressed (at high levels) in the preputial gland and testis, two tissues in which *Mup* genes are not expressed. The gene, when introduced into five of the seven lines, carried a copy of the *Escherichia coli supF* gene attached beyond the 3′ end of the HSV *tk* gene, but this did not affect the overall expression pattern. All of the lines were male sterile and expressed HSV thymidine kinase in the testis, but one line showed no activity in the liver, and another showed none in the preputial gland. Testicular expression is therefore the likely cause of sterility. Data are described which suggest that the causes of misexpression in the preputial gland and testis are different. Since expression in each tissue occurred in several lines, the structure of the hybrid gene must be responsible in each case. Five intensively studied lines showed at least four consistently different patterns of relative expression in preputial gland, testis, male liver, and female liver. These differences do not correlate in any way with the copy number of the foreign gene in the different lines and must be due to some other aspect of line-specific integration.

The mouse major urinary proteins (MUPs) are coded for by a family of about 35 genes clustered on chromosome 4 (3, 12, 23). Four groups of *Mup* genes have been identified, the largest of which are the group 1 genes and the probably inert group 2 pseudogenes (3, 12, 25, 35, 51; R. Al-Shawi, P. Ghazal, A. J. Clark, and J. O. Bishop, submitted for publication). Most group 1 genes are expressed exclusively in the liver, while a small subset are also expressed in the mammary glands of pregnant females (50, 52). Of the two smaller groups, the group 3 genes are also expressed in the liver, but under hormonal control different from the group 1 genes (35), and the group 4 genes are expressed in the lachrymal and salivary glands (50, 51).

Female mouse liver contains at least five times less MUP mRNA than male liver (23), although expression in females may be rather variable (I. McIntosh and J. O. Bishop, unpublished data). The difference in mRNA levels reflects different rates of transcription in the two sexes (18), and male levels of expression can be induced in females by administration of testosterone (14, 58). Relevant to the work presented here, the group 1 genes are regulated by circulating testosterone (14, 58). We are attempting to identify the regions in *Mup* genes which lead to their tissue specificity and hormone responses. Because liver cell lines which synthesize MUP are unknown, we are employing transgenic mice for this purpose. In the first instance we have studied the expression of a hybrid gene which contains as a reporter the herpes simplex virus type 1 thymidine kinase gene (HSV *tk*) attached to the putative promoter region of the group 1 *Mup* gene *BS6* (11, 13). The hybrid gene was expressed in the liver of transgenics, and in three of five lines it displayed a level of sexual dimorphism comparable to that of the *Mup* genes. Unexpectedly, it was also expressed consistently and at high levels in both preputial gland and testis. In all other tissues examined, RNA and protein products of the hybrid gene were absent, except that in one line there was expression in the lachrymal gland. Transgenic males were sterile, probably because of the presence of HSV thymidine kinase activity in the testis.

MATERIALS AND METHODS

Construction of hybrid genes. Plasmids were based on pSV2gpt (43), retaining the 341-base-pair (bp) *Pvu*II-*Hin*dIII simian virus 40 (SV40) enhancer-early promoter region and the 2,293-bp *Pvu*II-*Eco*RI portion of pBR322 containing the origin of replication and the β-lactamase gene. The SV40 enhancer in *cis* configuration is known to activate the *BS6* promoter in transfected rodent fibroblasts, allowing us to confirm before microinjection that the construct directs the synthesis of thymidine kinase. Between the *Hin*dIII site and the *Eco*RI site the following fragments were inserted (an asterisk shows that the site was blunt-ended with DNA

* Corresponding author.

polymerase I): an approximately 2.2-kilobase (kb) *Hin*dIII-*Sau*3A1 fragment of *Mup* gene *BS6* (13); seven nucleotides of polylinker (GATCCCC); and a 1,759-bp *Bgl*II*-*Bst*EII* fragment of HSV *tk* from plasmid pTK1 (59). The plasmid pSV-*BS6-tk-supF* was completed with a 10-bp *Sal*I*-*Bam*HI* fragment of the M13tg130 polylinker (31), a 400-bp *Alu*I-*Xba*I fragment of PiVX containing the *Escherichia coli supF* gene (49), and a 36-bp *Xba*I-*Eco*RI fragment of the M13tg130 polylinker. Plasmid pSV-*BS6-tk*-M was completed with a 57-bp *Sal*I*-*Eco*RI fragment of the polylinker. The fragments for microinjection were prepared by digestion with *Hin*dIII and *Kpn*I (*BS6-tk-supF*) or *Hin*dIII alone (*BS6-tk*-M), preparative agarose electrophoresis, electroelution, purification with Elutips (Schleicher and Schuell), phenol extraction, and repeated ethanol precipitation, and were dissolved in double-distilled water at a final concentration of 1.5 μg/ml.

Transgenic animals and lines. C57BL/6J × CBA F_1 females were superovulated by treatment with pregnant mare's serum and human chorionic gonadotropin (27) and mated with F_1 males. About 2 pl of DNA solution was injected into the more convenient pronucleus of each egg, and eggs were incubated in culture medium overnight. Two-cell embryos were introduced into pseudopregnant MF1 foster mothers. Transgenic mice were identified by Southern blot analysis of tail DNA. Transgenic females were mated to C57BL/6J × CBA F_1 males. Copy number estimates of the transgene were determined from Southern blot data.

Assessment of fertility. Male mice were housed with two female mice each, for a minimum of 7 weeks. Females were checked daily until vaginal plugs were seen. The fertility of an equal number of nontransgenic male siblings was tested in the same way, and all were found to be fertile.

Thymidine kinase assay. Thymidine kinase assays were carried out as described (6), but 0.4 mM TTP was added to inhibit endogenous thymidine kinase (29). Each determination involved equal portions of duplicate assays taken at three time intervals. The unit of thymidine kinase activity is the amount of enzyme which catalyzes the formation of 1 pmol of TMP in 1 min. Protein was determined by the Bradford method (5).

Northern blots and run-on transcription. RNA was prepared by a guanidinium thiocyanate method (9), and Northern blot analysis was performed as described (54) using Hybond-N (Amersham) and random-primed probe synthesis to a specific activity of approximately 10^9 cpm/μg. Probe A, the *Bam*HI-to-*Bam*HI HSV fragment of pTK1 (59), overlaps the HSV portion of the two constructs completely. Probe B, an 840-bp *Pst*I fragment of pTK1, overlaps 806 bp between the point of fusion with the *BS6* promoter region and a *Pst*I site internal to the *tk* coding region. Marker RNA was made by run-off transcription of plasmid DNA containing a 1,922-bp *Eco*RI-*Pvu*II fragment of pTK1 inserted in the polylinker region of pT7.1 (United States Biochemicals) 10 bp downstream of the T7 RNA polymerase transcription initiation site. The plasmid was digested separately with *Acc*I, *Ban*I, *Sma*I, and *Nar*I, and each fragment was transcribed in the presence of low-specific-activity [^{3}H]UTP to generate RNA fragments 434, 1,009, 1,306, and 1,816 nucleotides long. After recovery, these were combined to make a set of markers. Run-on transcription assays were performed as described (54). The plasmid target sequences were pTK1, MUP11 (10), and LVB6 (54).

RESULTS

We linked 2.2 kb of 5′-*BS6* sequence to HSV *tk*, with the junction point between the *BS6* cap site and the HSV *tk* initiation codon (Fig. 1). To facilitate the rescue of the hybrid gene from the genomic DNA of transgenic mice, the *BS6-tk-*

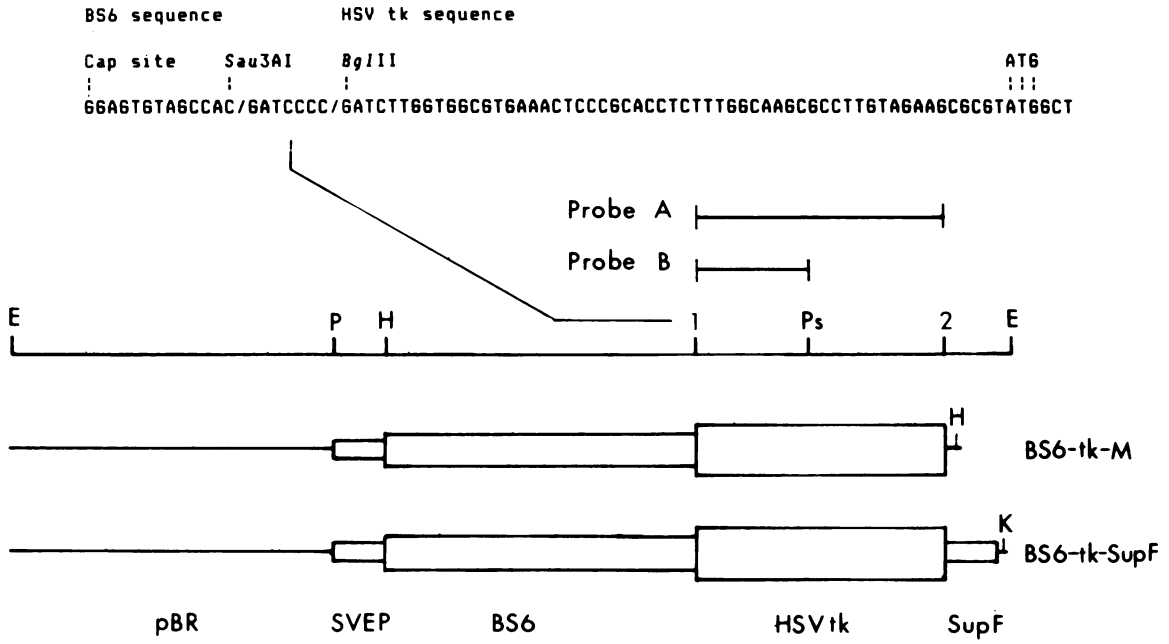

FIG. 1. Structures of hybrid genes. The restriction site map shows key sites as follows: E, *Eco*RI; P, *Pvu*II; Ps, *Pst*I; H, *Hin*dIII; K, *Kpn*I; 1, the *Sau*3A1-polylinker-*Bgl*II fusion between the *BS6* promoter and the HSV *tk* sequence, shown in the nucleotide sequence above; 2, the *Bst*EII-*Sal*I fusion between the HSV sequence and the M13tg130 polylinker. The *BS6-tk*-M fragment was separated from SV40 and vector sequences by digestion with *Hin*dIII, and the *BS6-tk-supF* fragment was separated by digestion with *Hin*dIII and *Kpn*I. For other details see the text.

TABLE 1. Transmission of foreign genes from G_0 to G_1 transgenics

G_0 line	Sex	G_1 offspring	
		Total	Transgenic (%)
40[a]	F	44	4 (9)
46[a]	F	58	15 (26)
58[a]	F	31	11 (35)
62	F	18	0 (0)
64[a]	F	21	6 (29)
78[a]	F	26	13 (50)
66	M	49	0 (0)
79	M	31	0 (0)
48	M	Sterile	
49	M	Sterile	
51	M	Sterile	

[a] Founders of transgenic lines.

supF construct contains the bacterial amber suppressor tRNA gene *supF* (19). This hybrid gene, freed from the SV40 and vector sequences, was microinjected into the pronuclei of fertilized mouse eggs. Of 40 live-born pups, 11 were identified as transgenic. From these, five "lines" were established, all from G_0 females (Table 1). Here a line is a group of male and female mice descended from the same G_0 female and containing the same transgenic insertion of foreign DNA.

Expression of the hybrid gene in liver, testis, and preputial gland. Expression of the hybrid gene was studied by probing Northern blots with probes specific for HSV *tk* and by assaying HSV thymidine kinase activity in tissue homogenates. Group 1 MUP mRNA is present in male and female liver and also in small amounts in the prelactation mammary gland, but not in other tissues (25, 50). The foreign HSV *tk* mRNA and thymidine kinase activity, however, were consistently present not only in male and female liver but also in testis and in preputial glands.

The expected size of a transcript running from the *BS6* cap site to the *tk* polyadenylation site is 1.3 kb. An RNA of this size was detected in male liver RNA from four of five lines, in testis RNA of five of five lines, and in male preputial gland RNA of four of five lines. The amount of this RNA is lowest in liver and highest in testis (Fig. 2). Each tissue contains a second smaller RNA that reacts with the *tk* probe to the same (testis) or a lesser (liver, preputial gland) extent than the 1.3-kb RNA. The smaller liver and testis RNAs are about 0.9 kb, and the preputial gland RNA is about 1 kb in size. Figure 2 was obtained after hybridization with probe *tk*-A (Fig. 1), which spans the entire HSV *tk* mRNA and the 3′-flanking region. When blots were hybridized with the shorter probe *tk*-B, which corresponds to the 5′ end of the HSV *tk* mRNA, the signal strength of the shorter liver and testis RNAs was less relative to the 1.3-kb RNA. This is consistent with transcriptional initiation of these RNAs downstream of the cap site, a phenomenon previously observed in cell lines carrying integrated HSV *tk* genes (47). The lengths of the smaller RNAs and their signal intensities, relative to the 1.3-kb RNA, are consistent within each transgenic line. No RNA that reacted with the HSV *tk* probe was detected in male lachrymal gland, salivary gland, spleen, heart, kidney, muscle, or brain.

The five transgenic lines were compared more intensively by means of thymidine kinase assays (Table 2). Tissues of three G_0 males were also examined in this way. In our hands the addition of 0.4 mM TTP to the assay (29) inhibited endogenous thymidine kinase activities in different tissues by 93 to 97%, and the residual endogenous activity in each tissue was quite consistent. The same amount of TTP inhibited HSV thymidine kinase by about 50%, so that even relatively low levels could be measured quite accurately. In most cases in which both mRNA determinations and enzyme activity measurements were made, the two were in broad agreement. Exceptions were the detection of low enzyme levels but no mRNA in line 64 female liver (see Table 4) and line 58 lachrymal gland (Table 2). In particular, the enzyme assays confirmed the expression of the foreign gene in the liver of four of five lines and, at higher levels, in the preputial glands of four of five lines and testes of all five lines (Table 3).

If the *supF* gene which is incorporated at the 3′ end of the hybrid gene were contributing to the expression pattern of the gene, its usefulness would be seriously reduced. To examine this possibility, the *BS6-tk*-M hybrid gene, which lacks the *supF* DNA fragment (Fig. 1), was also introduced into mice. Five transgenic G_0 animals were obtained, and two transgenic lines were established from G_0 females. Thymidine kinase assays again showed expression in liver and higher expression in testis and preputial gland (Table 2). The similarity of these results to those obtained with the construct which contains *supF* indicates that the *supF* gene does not influence the expression of this hybrid gene.

Two features of the expression of the foreign gene are line specific. (i) Sporadic or occasional expression: we observed thymidine kinase expression in the lachrymal gland of one of four lines tested, and the absence of preputial gland expression in one of five lines and of liver expression in one of five lines (Table 2). (ii) Relative levels of expression: the relative levels of expression in different tissues are also line specific (Table 3). With the exception of lines 58 and 78, each line shows a different expression pattern. Line-specific features of expression in transgenics most probably relate to the different chromosomal integration events which characterize the different lines.

Sexually dimorphic expression of the *BS6-tk* hybrid gene. In three of five transgenic lines, male liver showed higher levels

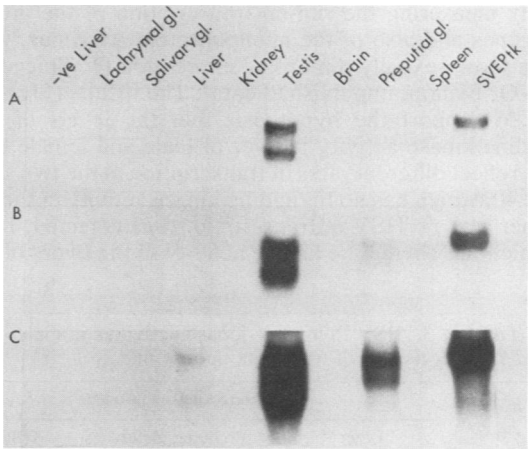

FIG. 2. HSV *tk* RNA in tissues of a transgenic mouse. Total cellular RNA from tissues of an adult male mouse of line 46 was applied to the gel, 30 μg to each track except for 10 μg of preputial gland RNA. The filter was hybridized with probe A and exposed for (A) 24 h and (B and C) 3 days. B and C are two printings of the 3-day exposure. SVEPtk, RNA from BHKtk$^-$ cells stably transformed with a plasmid containing HSV *tk* linked directly to the SV40 enhancer-early promoter region; −ve liver, nontransgenic liver RNA.

TABLE 2. HSV thymidine kinase activities in tissues of male transgenic and control mice

Tissue	Transgenic (*BS6-tk-supF*)				Transgenic (*BS6-tk*-M)				Controls[a]: mean activity ± S.E. (no. of mice assayed)
	Lines[b]	G_0[c]	Total[d]	Mean activity[e]	Lines[b]	G_0[c]	Total	Mean activity[e]	
Liver	4/5	2/3	6/8	3.8	2/2	1/1	3/3	0.8	0.015 ± 0.007 (12)
Preputial gland	4/5	2/3	6/8	25	2/2	1/1	3/3	79	0.103 ± 0.033 (10)
Testis	5/5	2/3	7/8	64	2/2	1/1	3/3	119	0.069 ± 0.028 (8)
Lachrymal gland	1/3	0/1	1/4	2.1	0/1	ND	0/1		0.023 ± 0.008 (4)
Salivary gland	0/3	0/1	0/4		0/1	ND	0/1		0.008 ± 0.003 (6)
Brain	0/3	0/3	0/6		0/1	ND	0/1		0.270 ± 0.029 (10)
Kidney	0/5	0/2	0/7		0/1	0/1	0/2		0.051 ± 0.012 (21)
Muscle	0/3	ND	0/3		0/1	ND	0/1		0.009 ± 0.002 (4)

[a] Nontransgenic littermates.
[b] Lines founded from G_0 females; lines positive/lines tested.
[c] G_0 males, individuals positive/individuals tested. ND, Not determined.
[d] One G_0 male showed no activity in any tissue.
[e] Thymidine kinase activity, units per milligram of protein, mean of line means and G_0 values (positive lines and G_0s only), not weighted for numbers of determinations per line mean. Among the G_0 males there are potential mosaics, which will depress the average values. Zero values were not significantly different from endogenous values measured under the same conditions.

of HSV thymidine kinase activity than female liver (Table 4). The two exceptions were line 40, which shows no activity in liver of either sex, and line 64, in which male and female liver have similar activity levels. Of the three lines with sexually dimorphic HSV *tk* activity, the male-to-female ratio of line 46 was about 10, while the ratio in lines 58 and 78 was about 4. These ratios compare with a ratio of 5 to 10 in liver MUP mRNA levels (23; McIntosh and Bishop, in preparation).

As noted above, HSV *tk* RNA was present in male liver RNA of four of five lines (lines 46, 58, 64, and 78). HSV *tk* RNA was detected in liver of line 58 females but not in liver of line 46 or 64 females, presumably because of the low levels of RNA present. (Line 78 females have not been examined.)

The different levels of MUP mRNA in male and female liver reflect different transcription rates (18). It was therefore of interest to determine by run-on transcription measurements whether the different expression levels of the transgene in males and females are also due to differential transcription. The experiments were controlled by simultaneously measuring the run-on transcription of the group 1 *Mup* genes and also of the apolipoprotein A1 genes, which do not show sexually dimorphic expression (T. Spiegelberg and J. O. Bishop, unpublished data). The results (Fig. 3 and Table 5) support the hypothesis that the levels of HSV thymidine kinase activity in liver of male and female transgenics reflect different rates of transcription in the two sexes. In line 40, which has no thymidine kinase activity in the liver of either sex, no HSV *tk* transcription was detected. In line 46, which has thymidine kinase activity in the livers of both sexes, but at a much lower level in females, transcription was detected in male but not female liver nuclei. In lines 58 and 78, which have a male/female liver thymidine kinase activity ratio of about 4, the male/female run-on transcription ratios were 6.5 and 2.5, respectively. Line 46 contains about 80 tandemly arranged copies of the hybrid gene per cell, while lines 58 and 78 contain about 3 and 1 copies, respectively (Table 3). Transcription rate, in common with enzymatic activity, is clearly independent of copy number.

The *Mup* gene target sequence used in these experiments is expected to react with all of the group 1 *Mup* gene transcripts. Our best estimate of the level of expression of *BS6* is that it accounts for 25% or less of total *Mup* gene mRNA in male liver (McIntosh and Bishop, in preparation). By an independent estimate based on cDNA cloning frequency, an mRNA identical to *BS6* mRNA has been shown to be very abundant in male liver mRNA (51). In the male transgenics the rate of run-on transcription of HSV *tk* is about 15% as high as that of all of the *Mup* genes taken together (Table 5). We recall that a proportion of the liver HSV *tk* mRNA is unexpectedly small in size (Fig. 2). How much of the primary transcript corresponds to this smaller mRNA is not known, and consequently a rigorous quantitative interpretation of the run-on data is not possible at this time. However, they do suggest that the rate of transcription of the hybrid gene in line 58 and line 78 liver is comparable to that of the endogenous *BS6* gene.

Testosterone induction of HSV thymidine kinase activity. We have observed considerable variation in expression between individuals within each transgenic line (see stan-

TABLE 3. HSV thymidine kinase activities in male tissues of transgenic mouse lines

Line	Copy no.[a]	Thymidine kinase[b] in tissue		
		Liver	Preputial gland	Testis
40	10	0.05 ± 0.04 (4)	12.0 ± 0.39 (2)	140 ± 6.7 (3)
46	80	1.9 ± 0.32 (8)	83.1 ± 19.9 (2)	139 ± 17.6 (3)
58	3	5.82 ± 1.15 (3)	9.9 ± 2.6 (3)	31 ± 10.5 (3)
64	2	1.52 ± 0.71 (2)	0.04 ± 0.03 (2)	48 ± 6.0 (2)
78	1	12.73 ± 1.85 (3)	32.1 ± 5.3 (3)	62 ± 8.4 (3)

[a] Approximate.
[b] Units per milligram of protein; means and standard errors. Parentheses indicate *n*.

TABLE 4. Sexually dimorphic expression of HSV thymidine kinase in liver of transgenic mouse lines

Line	Enzyme activity[a]		*t* test[b] (*P*)
	Male	Female	
40	0.05 ± 0.04 (4)	0 (3)	
46	1.9 ± 0.32 (8)	0.17 ± 0.16 (8)	<0.001
58	5.82 ± 1.15 (3)	1.49 ± 0.1 (2)	<0.1
64	1.52 ± 0.71 (2)	2.35 ± 0.32 (2)	
78	12.73 ± 1.85 (3)	2.80 ± 0.59 (3)	<0.01

[a] Units per milligram of protein; means and standard errors. Parentheses indicate *n*.
[b] Probability that male and female samples are drawn from the same population.

TABLE 5. Run-on transcription of *BS6-tk* and *Mup* genes in transgenic liver

Line	Transcription rate[a]			Thymidine kinase activity[b]		
	Male	Female	M/F	Male	Female	M/F
40	0	0		0	0	
46	0.15	0		1.9	0.17	10.1
58	0.13	0.02	6.5	5.8	1.5	3.8
78	0.2	0.08	2.5	12.7	2.8	4.3

[a] The data are based on densitometer readings of autoradiographs. Each value is the ratio of the HSV *tk* measurement to the *Mup* measurement obtained from male liver nuclei in the same experiment. Variation within and between experiments was compensated by normalizing the other measurements to the run-on value observed for apolipoprotein A1 with RNA from the same incubation of nuclei.

[b] Average thymidine kinase activity measurements from Table 4.

dard errors in Tables 3 and 4), which could complicate the interpretation of testosterone induction experiments. For this reason the induction experiments were carried out with females of line 46, which consistently show very low HSV thymidine kinase activity in the liver (Table 4). Testosterone pellets were implanted subcutaneously 12 to 14 days before the livers were analyzed. Control transgenic littermates were sham-operated at the same time. Testosterone treatment did not elicit any change in thymidine kinase activity in nontransgenic female liver. The results (Fig. 4A) clearly show that HSV thymidine kinase activity is induced by testosterone. Northern blot analysis of mRNA from induced and noninduced female liver showed induction of HSV *tk* RNA levels (Fig. 4B).

Liver HSV thymidine kinase activity in the induced females was on average 37% of the level in noninduced males, whereas in normal females MUP is induced to male levels (14, 58). This difference may relate to the very low HSV thymidine kinase levels in livers of noninduced line 46 females. For example, if, like the α_{2u}-globulin genes (36), *Mup* genes are repressed by estrogen, the hybrid genes in line 46 may be more sensitive to estrogen repression. Other explanations are of course possible. The results, however, do show clearly that in line 46 the expression of the hybrid gene is induced by testosterone.

Sterility of male transgenic mice that carry the BS6-*tk* hybrid gene. Three of five *BS6-tk-supF* G_0 males and two of three *BS6-tk*-M G_0 males were sterile. None of the fertile G_0 males transmitted the hybrid gene to their offspring. In addition, in seven of seven transgenic lines the males were invariably sterile. Two exceptional mice, one from line 58

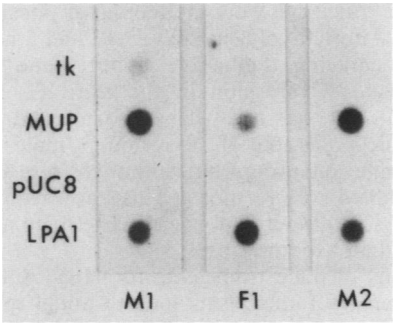

FIG. 3. Sex difference in run-on transcription rate of the *BS6-tk* hybrid gene in transgenic liver. M1, Transgenic male; F1, transgenic female; M2, nontransgenic male. Immobilized target sequences: tk, HSV *tk*; MUP, MUP11 cDNA; LPA1, apolipoprotein A1 cDNA.

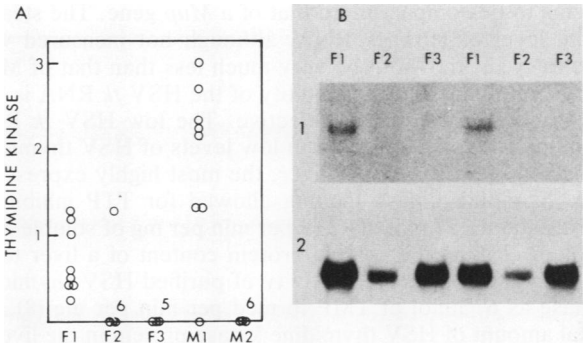

FIG. 4. Induction of the expression of the *BS6-tk* hybrid gene in line 46 female liver. (A) HSV thymidine kinase levels. F1, Transgenic females, testosterone induced; F2, transgenic females, sham operated; F3, nontransgenic females, testosterone induced; M1, transgenic males; M2, nontransgenic males. Thymidine kinase is expressed as picomoles of TMP formed per minute per milligram of soluble protein. (B) HSV *tk* RNA and MUP mRNA. Poly(A) RNA was partially purified by treating liver RNA with Hybond-mAP (Amersham), and 30 μg was applied to each track. The filter was (1) hybridized with probe B and then (2) stripped and rehybridized with group 1 MUP cDNA probe MUP11. F1, Transgenic females, testosterone induced; F2, transgenic females, sham operated; F3, nontransgenic females, testosterone induced.

and one from a *BS6-tk*-M line, each sired a single offspring of unknown transgenic status. No further offspring were sired despite repeated mating. These mice, and also all the completely sterile mice tested, plugged females in the normal way. The female transgenics exhibited normal fertility. While no HSV thymidine kinase was detected in ovaries (data not shown), expression in the follicles themselves would be obscured by the large excess of nongerminal tissue. The transgenic males have reduced sperm counts, and most of the spermatozoa are structurally abnormal to varying degrees. Thus sperm maturation seems to be impaired rather than absolutely blocked, and this is confirmed by the two offspring sired.

DISCUSSION

Consistent tissue-specific expression and sexual dimorphism. We have shown that a 2.2-kb group 1 *Mup* gene promoter region and cap site consistently direct synthesis of the HSV *tk* reporter gene to the liver, preputial gland, and testis. Expression in the liver mirrors the expression of the endogenous group 1 *Mup* genes; three of four lines showed differential expression in males and females, and when females of one of the lines were treated with testosterone, elevated levels of both HSV *tk* RNA and thymidine kinase activity resulted. Thus many or all of the *cis*-acting signals necessary for correct *Mup* expression in the liver appear to be present within the 2.2-kb promoter fragment. Run-on experiments showed that the different levels of HSV *tk* RNA and thymidine kinase activity in males and females are due at least in part to different rates of transcription. By analogy with the mechanism of action of other steroid hormones (61), the data suggest that testosterone, through its receptor, is acting on sequences within the 2.2-kb *BS6* promoter region to increase transcription. As yet it is not known whether the *cis*-acting signals responsible for the effects of thyroxine and growth hormone on MUP RNA synthesis in the liver (33, 54) are also present.

In the livers of lines 58 and 78, both of which carry a small number of foreign genes, the rate of HSV *tk* transcription

seems to be comparable to that of a *Mup* gene. The steady-state level of HSV *tk* RNA, although not measured with accuracy, is known to be very much less than that of MUP RNA, implying that the stability of the HSV *tk* RNA is less or that its processing is defective. The low HSV *tk* RNA content of liver is reflected in low levels of HSV thymidine kinase protein. In line 78 liver, the most highly expressing, the thymidine kinase (having allowed for TTP inhibition) forms about 25 pmol of TMP per min per mg of soluble liver protein. Taking the soluble protein content of a liver to be 100 mg and the specific activity of purified HSV thymidine kinase as 67 nmol of TMP formed per min per mg (8), the total amount of HSV thymidine kinase protein in the liver is about 0.04 mg. This may be compared with a MUP production rate of 10 mg per male mouse per day (58).

Consistent misexpression. It is frequently found that a foreign gene is correctly expressed in transgenic mice, sometimes to the extent that the timing of expression is correct (for example, see references 1, 22, 34, 40, 46, and 57). Misexpression is most often observed with hybrid genes like the *BS6-tk* fusion gene described here. So long as misexpression is consistent, its study can lead to insights into mechanisms of gene regulation. Also, insight into misexpression is important in relation to attempts to direct the synthesis of proteins to novel tissues for commercial purposes (21, 53).

The consistent expression of the *BS6-tk* gene in preputial gland and testis is unexpected, since the *Mup* genes are not expressed in either tissue. While the level of expression both of RNA and of thymidine kinase activity in both tissues is higher, sometimes much higher, than in liver, we have not measured transcription rates in these tissues, and the differences may be due to RNA processing or stability differences rather than to transcription rates themselves.

Consistent misexpression of foreign genes has been observed before (2, 4, 38, 42, 45, 56). If, as seems likely, it is due to changes in transcriptional behavior rather than increased RNA stability, it may be explained in either of two ways. Either (i) the hybrid gene lacks *cis*-acting "silencer" sequences which are required for repression of the endogenous gene in preputial gland and testis and which are located outside the 2.2-kb promoter region, or (ii) it contains newly created sequences or a new combination of sequences which activate its transcription in these tissues (56). We are presently attempting to distinguish between these alternatives.

The α_{2u}-globulins, the rat homologs of the *Mup* genes, show a similar pattern of gene expression (37, 39). A clear difference in the expression of the rat and mouse genes, however, is that α_{2u}-globulin is expressed at a high level in male and female preputial gland, while *Mup* is not expressed in the preputial gland (24, 39). However, an α_{2u}-globulin gene is expressed as a foreign gene in the preputial glands of transgenic mice (16). One interpretation of this observation is that the natural rat gene lacks a silencer sequence which is present in the mouse genes, resulting in its expression in mouse preputial glands despite the presence in the mouse tissue of the corresponding *trans*-acting repressor protein (16). The *BS6-tk* hybrid gene was consistently expressed not only in male and female preputial glands but also in testis. If misexpression in preputial gland and testis were due to the same cause, we might expect there to be α_{2u}-globulin expression in rat testis. However, no α_{2u}-globulin mRNA could be detected by Northern blotting (data not shown). Appropriate controls showed that the testis RNA used in this experiment was not degraded. This observation suggests either that the misexpression of the hybrid *Mup* gene in preputial gland and testis has different causes, or else, if there is a common cause, that it is independent of the causes of α_{2u}-globulin expression in rat and mouse preputial glands. Two observations suggest that different causes bring about the misexpression of the hybrid gene in the preputial gland and testis. First, expression in the testis occurs in a transgenic line with no preputial gland expression. Second, the smaller preputial gland and testis RNAs differ from each other in size and in their hybridization to different probes.

Despite some variability between mice within each transgenic line, the pattern of expression levels in liver, preputial gland, and testis is line specific. Line-specific effects are assumed to be due to the uniqueness of different chromosomal integration events. These events differ in several ways. Different sites may contain different numbers and different arrangements (head-to-head, head-to-tail, tail-to-tail) and rearrangements of the integrated foreign genes. In addition, flanking DNA sequences at different chromosomal sites of integration are thought possibly to influence transcription of the foreign genes through "position effects." In the present case no correlation has been detected between copy number and expression in any tissue (Table 3), a common observation in transgenics (17, 44). The question of whether the different patterns of expression are due to arrangements, rearrangements, or position effects has not been addressed. Lines 58, 64, and 78, all of which contain a small number of integrated gene copies, provide convenient models with which to distinguish these causes.

Transgenic male sterility is due to HSV thymidine kinase activity in the testis. All of the established lines show both consistent male sterility and a high level of HSV thymidine kinase in the testis. In contrast, line 40 shows no liver HSV thymidine kinase activity and line 64 shows no preputial gland HSV thymidine kinase activity, although both lines are consistently male sterile. Thus testis expression of HSV thymidine kinase is the likely cause of male sterility, and both liver and preputial gland expression can be ruled out as the sole cause.

Minor exceptions to the sterility of transgenic males were observed. First, three G_0 males (two carrying *BS6-tk-supF* and one with *BS6-tk*-M) sired offspring, none of which were transgenic. G_0 male 79 had no testis HSV thymidine kinase activity, and indeed showed no thymidine kinase activity in any tissue. This male presumably carried transcriptionally inert copies of the hybrid gene and in addition was a mosaic with nontransgenic germ cells. G_0 male 66 had a very low level of testis HSV thymidine kinase activity (60% over the endogenous level, after discounting TTP inhibition). This male may be explained in either of two ways. He may have had a mosaic germ line, with the implication that germ cells carrying the transgene were ineffectual or possibly very few in number. Alternatively, he may have had a nontransgenic germ line, carrying the active hybrid gene in testicular somatic tissue. Expression in the third G_0 male, which carries *BS6-tk*-M, has not yet been examined. Secondly, 2 G_1 transgenic males, out of 21 G_1 and G_2 males tested, each sired a single offspring, but although repeatedly mated thereafter failed to sire more. This shows that the sterile males form some functional sperm. The mice are oligospermic rather than aspermic.

Some lines of mice transgenic for HSV *tk* linked to the mouse mammary tumor virus long terminal repeat express HSV thymidine kinase in the testis (48) and are also sterile (S. Ross and D. Solter, personal communication). However, at least one fertile mouse line which expresses HSV thymidine kinase in the testis has been reported (32). In this case

the HSV *tk* gene was under the control of its natural promoter. Thus expression of HSV thymidine kinase in the testis does not invariably lead to sterility. One possible explanation is that lower expression levels do not cause sterility; the HSV thymidine kinase activity level in the fertile line is about 3 times the endogenous level (32), whereas the lowest level among our sterile lines (line 58) is about 12 times the endogenous level. However, since the testis contains several cell types with different functions and since sperm differentiation is a slow process accompanied by changes in both the germ cells and accessory cells (60), an alternative explanation is that sterility is linked to expression in particular cell types or at particular developmental stages. This explanation is especially plausible because HSV *tk* expression is under the control of different promoters in the different experiments. Furthermore, the expression of the mouse mammary tumor virus *tk* hybrid gene which confers sterility may, like that of *BS6-tk*, be induced by testosterone (7, 15).

Given that sterility is due to HSV thymidine kinase expression in one or more testis cell types, there are several ways in which the enzyme could have its effect. First, its substrate specificity is different from that of the cellular enzyme, and it efficiently phosphorylates deoxycytidine (28). Second, the high levels of phosphorylated deoxynucleosides produced by high levels of the enzyme will inhibit the normal pathway of pyrimidine phosphate metabolism (30). Third, HSV thymidine kinase may be active at times in the cell cycle when the cellular enzyme is normally repressed (26, 41, 55). Any of these could perturb cellular metabolism, primarily of nucleic acid precursors, but with secondary effects on related pathways. Transgenic mice expressing a mutant form of dihydrofolate reductase, which is also involved in pyrimidine metabolism, show reduced growth, reduced female fertility, and morphological defects (20).

ACKNOWLEDGMENTS

We thank Neil Wilkie for providing pTK1 and BHK cells; David Finnegan for PiVX; Sam Martin for implanting female mice with testosterone pellets; Sheila Barton and Azim Surani for advice on oviduct transfers; and Anne Walker, Roberta Wallace, Melville Richardson, Ann Duncan, Helen McIlroy, and Garry Brown for their assistance.

The work was supported by the AFRC and Medical Research Council.

LITERATURE CITED

1. **Behringer, R. R., R. E. Hammer, R. L. Brinster, R. D. Palmiter, and T. M. Townes.** 1987. Two 3′ sequences direct adult erythroid-specific expression of human beta-globin genes in transgenic mice. Proc. Natl. Acad. Sci. USA **84**:7056–7060.
2. **Behringer, R. R., J. J. Peschon, A. Messing, C. L. Gartside, S. D. Hauschka, R. D. Palmiter, and R. L. Brinster.** 1988. Heart and bone tumors in transgenic mice. Proc. Natl. Acad. Sci. USA **85**:2648–2652.
3. **Bishop, J. O., A. J. Clark, P. M. Clissold, S. Hainey, and U. Francke.** 1982. Two main groups of mouse major urinary protein genes, both largely located on chromosome 4. EMBO J. **1**:615–620.
4. **Botteri, F. M., H. van der Putten, D. F. Wong, C. A. Sauvage, and R. M. Evans.** 1987. Unexpected thymic hyperplasia in transgenic mice harboring a neuronal promoter fused with simian virus 40 large T antigen. Mol. Cell. Biol. **7**:3178–3184.
5. **Bradford, M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Brinster, R. L., Y. H. Chen, and M. Trumbauer.** 1981. Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. Cell **27**:223–231.
7. **Cato, A. C. B., D. Henderson, and H. Ponta.** 1987. The hormone responsive element of the mouse mammary tumor virus DNA mediates the progestin and androgen induction of transcription in the proviral long terminal region. EMBO J. **6**:363–368.
8. **Cheng, Y.-C., and M. Ostrander.** 1976. Deoxythymidine kinase induced in HeLa TK^- cells by HSV type I and type II. J. Biol. Chem. **251**:2605–2610.
9. **Chirgwin, J. M., A. E. Przybyla, R. J. Macdonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
10. **Clark, A. J., A. Chave-Cox, X. Ma, and J. O. Bishop.** 1985. Analysis of mouse major urinary protein genes: variation between the exonic sequences of Group 1 genes and a comparison with an active gene outwith Group 1 both suggest that gene conversion has occurred between MUP genes. EMBO J. **4**: 3167–3171.
11. **Clark, A. J., P. M. Clissold, R. Al-Shawi, P. Beattie, and J. O. Bishop.** 1984. Structure of mouse major urinary protein genes: different splicing configurations in the 3′-non-coding region. EMBO J. **3**:1045–1052.
12. **Clark, A. J., P. M. Clissold, and J. O. Bishop.** 1982. Variation between mouse major urinary protein genes isolated from a single inbred line. Gene **18**:221–230.
13. **Clark, A. J., P. Ghazal, R. W. Bingham, D. Barrett, and J. O. Bishop.** 1985. Sequence structures of a mouse major urinary protein gene and pseudogene compared. EMBO J. **4**:3159–3165.
14. **Clissold, P. M., S. Hainey, and J. O. Bishop.** 1984. Messenger RNAs coding for mouse major urinary proteins are differentially induced by testosterone. Biochem. Genet. **22**:379–387.
15. **Dabre, P., M. Page, and R. J. B. King.** 1986. Androgen regulation by the long terminal repeat of mouse mammary tumor virus. Mol. Cell. Biol. **6**:2847–2854.
16. **da Costa Soares, V., R. M. Gubits, P. Feigelson, and F. Costantini.** 1987. Tissue-specific and hormonally regulated expression of a rat α_{2u}-globulin gene in transgenic mice. Mol. Cell. Biol. **7**: 3749–3758.
17. **Davis, B. P., and R. J. MacDonald.** 1988. Limited transcription of rat elastase I transgene repeats in transgenic mice. Genes Dev. **2**:13–22.
18. **Derman, E.** 1981. Isolation of a cDNA clone for mouse urinary proteins: age- and sex-related expression of mouse urinary protein genes is transcriptionally controlled. Proc. Natl. Acad. Sci. USA **78**:5425–5429.
19. **Goldfarb, M., K. Shimizu, M. Perucho, and M. Wigler.** 1982. Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells. Nature (London) **296**:404–409.
20. **Gordon, J. W.** 1986. A foreign dihydrofolate reductase gene in transgenic mice acts as a dominant mutation. Mol. Cell. Biol. **6**: 2158–2167.
21. **Gordon, K., E. Lee, J. A. Vitale, A. E. Smith, H. Westphal, and L. Hennighausen.** 1987. Production of human tissue plasminogen activator in transgenic mouse milk. Bio/Technology **5**:1183–1187.
22. **Hanahan, D.** 1985. Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature (London) **315**:115–122.
23. **Hastie, N. D., W. A. Held, and J. J. Toole.** 1979. Multiple genes coding for the androgen-regulated major urinary proteins of the mouse. Cell **17**:449–457.
24. **Held, W. A., and J. F. Gallagher.** 1985. Rat alpha-2u globulin mRNA expression in the preputial gland. Biochem. Genet. **32**: 281–290.
25. **Held, W. A., J. F. Gallagher, C. M. Hohman, N. J. Kuhn, B. M. Sampsell, and R. G. Hughes, Jr.** 1987. Identification and characterization of functional genes encoding the mouse major urinary proteins. Mol. Cell. Biol. **7**:3705–3712.
26. **Hofbauer, R., E. Muellner, C. Seiser, and E. Wintersberger.** 1987. Cell cycle regulated synthesis of stable mouse thymidine kinase mRNA is mediated by a sequence within the cDNA.

Nucleic Acids Res. **15**:741–752.
27. **Hogan, B. L., F. Costantini, and E. Lacy.** 1986. Manipulating the mouse embryo. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Jamieson, A. T., G. A. Gentry, and J. A. Subak-Sharpe.** 1974. Induction of both thymidine and deoxycytidine kinase activity by herpes virus. J. Gen. Virol. **24**:465–480.
29. **Jamieson, A. T., and J. H. Subak-Sharpe.** 1974. Biochemical studies on the herpes simplex virus specified deoxypyrimidine kinase activity. J. Gen. Virol. **24**:481–492.
30. **Kelley, W. N.** 1983. Hereditary orotic aciduria, p. 1202–1226. *In* J. B. Stanbury, J. B. Wyngarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown (ed.), The metabolic basis of inherited disease. McGraw-Hill, New York.
31. **Kieney, M. P., R. Lathe, and J. P. Lecoq.** 1983. New versatile cloning and sequencing vectors based on bacteriophage M13. Gene **26**:91–99.
32. **Kiessling, U., K. Becker, M. Strauss, J. Schoeneich, and E. Giessler.** 1986. Rescue of a tk- plasmid from transgenic mice reveals its episomal transmission. Mol. Gen. Genet. **204**:328–333.
33. **Knopf, J. L., J. F. Gallagher, and W. A. Held.** 1983. Differential multihormonal regulation of the mouse urinary protein gene family in the liver. Mol. Cell. Biol. **3**:2232–2240.
34. **Krumlauf, R., R. E. Hammer, S. M. Tilghman, and R. L. Brinster.** 1985. Developmental regulation of alpha-fetoprotein genes in transgenic mice. Mol. Cell. Biol. **5**:1639–1648.
35. **Kuhn, N. J., M. Woodworth-Gutai, K. W. Gross, and W. A. Held.** 1984. Subfamilies of the mouse major urinary protein (MUP) multi-gene family: sequence analysis of cDNA clones and differential regulation. Nucleic Acids Res. **12**:6073–6090.
36. **Kurtz, D. T., and P. Feigelson.** 1978. Multihormonal control of the messenger RNA for the hepatic protein alpha2u-globulin, p. 433–455. *In* G. Litvak (ed.), Biochemical actions of hormones, vol. 5. Academic Press, Inc., New York.
37. **Laperche, Y., K. R. Lynch, K. P. Dolan, and P. Feigelson.** 1983. Tissue-specific control of alpha2u-globulin gene expression: constitutive synthesis in the submaxillary gland. Cell **32**:453–460.
38. **Low, M. J., R. M. Lechan, R. E. Hammer, R. L. Brinster, J. F. Habener, G. Mandel, and R. H. Goodman.** 1986. Gonadotroph-specific expression of metallothionein fusion genes in pituitaries of transgenic mice. Science **231**:1002–1004.
39. **MacInnes, J. I., E. S. Nozik, and D. T. Kurtz.** 1986. Tissue-specific expression of the rat alpha$_{2u}$ globulin gene family. Mol. Cell. Biol. **6**:3563–3567.
40. **Magram, J., K. Chada, and F. Costantini.** 1985. Developmental regulation of an adult beta-globin gene in transgenic mice. Nature (London) **315**:338–340.
41. **Merrill, G. F., S. D. Hauschka, and S. L. McKnight.** 1984. tk enzyme expression in differentiating muscle cells is regulated through an internal segment of the cellular *tk* gene. Mol. Cell. Biol. **4**:1777–1784.
42. **Messing, A., H. Y. Chen, R. D. Palmiter, and R. L. Brinster.** 1983. Peripheral neuropathies, hepatocellular carcinomas and islet cell adenomas in transgenic mice. Nature (London) **316**:461–463.
43. **Mulligan, R. C., and P. Berg.** 1980. Expression of a bacterial gene in mammalian cells. Science **209**:1422–1427.
44. **Overbeek, P. A., S.-P. Lai, K. R. Van Quill, and H. Westphal.** 1986. Tissue-specific expression in transgenic mice of a fused gene containing RSV terminal sequences. Science **231**:1574–1576.
45. **Palmiter, R. D., and R. L. Brinster.** 1986. Transgenic mice. Annu. Rev. Genet. **20**:465–499.
46. **Peschon, J. J., R. R. Behringer, R. L. Brinster, and R. D. Palmiter.** 1987. Spermatid-specific expression of protamine 1 in transgenic mice. Proc. Natl. Acad. Sci. USA **84**:5316–5319.
47. **Roberts, J. M., and R. Axel.** 1982. Gene amplification and gene correction in somatic cells. Cell **29**:109–119.
48. **Ross, S. R., and D. Solter.** 1985. Glucocorticoid regulation of mouse mammary tumor virus sequences in transgenic mice. Proc. Natl. Acad. Sci. USA **82**:5880–5884.
49. **Seed, B.** 1983. Purification of genomic sequences from bacteriophage libraries by recombination and selection *in vivo*. Nucleic Acids Res. **11**:2427–2445.
50. **Shahan, K., M. Denaro, M. Gilmartin, Y. Shi, and E. Derman.** 1987. Expression of six mouse major urinary protein genes in the mammary, parotid, sublingual, submaxillary, and lachrymal glands and in the liver. Mol. Cell. Biol. **7**:1947–1954.
51. **Shahan, K., M. Gilmartin, and E. Derman.** 1987. Nucleotide sequences of liver, lachrymal, and submaxillary gland mouse major urinary protein mRNAs: mosaic structure and construction of panels of gene-specific synthetic oligonucleotide probes. Mol. Cell. Biol. **7**:1938–1946.
52. **Shaw, P. H., W. A. Held, and N. D. Hastie.** 1983. The gene family for major urinary proteins: expression in several secretory tissues of the mouse. Cell **32**:755–761.
53. **Simons, J. P., I. Wilmut, A. J. Clark, A. L. Archibald, J. O. Bishop, and R. Lathe.** 1988. Gene transfer into sheep. Bio/Technology **6**:179–183.
54. **Spiegelberg, T., and J. O. Bishop.** 1988. Tissue-specific gene expression in mouse hepatocytes cultured in growth-restricting medium. Mol. Cell. Biol. **8**:3338–3344.
55. **Stubblefield, E., and G. C. Mueller.** 1965. Thymidine kinase activity in synchronised HeLa cells. Biochem. Biophys. Res. Commun. **20**:535–538.
56. **Swanson, L. W., D. M. Simmons, J. Arriza, R. Hammer, R. Brinster, M. G. Rosenfeld, and R. M. Evans.** 1985. Novel developmental specificity in the nervous system of transgenic animals expressing growth hormone fusion genes. Nature (London) **31**:363–366.
57. **Swift, G. H., R. E. Hammer, R. J. MacDonald, and R. L. Brinster.** 1984. Tissue-specific expression of the rat pancreatic elastase 1 gene in transgenic mice. Cell **38**:639–646.
58. **Szoka, P., and K. Paigen.** 1978. Regulation of mouse major urinary protein production by the *Mup-a* gene. Genetics **90**:597–612.
59. **Wilkie, N. M., J. B. Clements, W. Boll, N. Mantei, D. Lonsdale, and C. Weissmann.** 1979. Hybrid plasmids containing an active thymidine kinase gene of herpes simplex virus type I. Nucleic Acids Res. **7**:859–877.
60. **Willison, K., and A. Ashworth.** 1987. Mammalian spermatogenic gene expression. Trends Genet. **12**:351–355.
61. **Yamamoto, K. R.** 1985. Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. **19**:209–252.